

Supporting Information

Ruthmycin, a New Tetracyclic Polyketide from *Streptomyces* sp. RM-4-15

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Contents	Page
Experimental Methods and Materials	S2
Supplemental References	S4
Figure S1. Antifungal disc diffusion assay	S5
Figure S2. APCI-MS (positive and negative mode) of ruthmycin (1)	S6
Figure S3. HR-ESI-MS (positive mode) of ruthmycin (1)	S7
Figure S4. ¹ H-NMR (Acetone- <i>d</i> ₆ , 400 MHz) of ruthmycin (1)	S8
Figure S5. ¹ H-NMR (DMSO- <i>d</i> ₆ , 500 MHz) of ruthmycin (1)	S9
Figure S6. ¹ H-NMR (CDCl ₃ , 500 MHz) of ruthmycin (1)	S10
Figure S7. ¹ H-NMR (CD ₃ OD, 500 MHz) of ruthmycin (1)	S11
Figure S8. ¹³ C-NMR (Acetone- <i>d</i> ₆ , 100 MHz) of ruthmycin (1)	S12
Figure S9. ¹³ C-NMR (DMSO- <i>d</i> ₆ , 100 MHz) of ruthmycin (1)	S13
Figure S10. HSQC spectrum (Acetone- <i>d</i> ₆ , 400 MHz) of ruthmycin (1)	S14
Figure S11. HMBC spectrum (Acetone- <i>d</i> ₆ , 400 MHz) of ruthmycin (1)	S15
Figure S12. ROESY spectrum (Acetone- <i>d</i> ₆ , 400 MHz) of ruthmycin (1)	S16
Figure S13. ROESY spectrum (DMSO- <i>d</i> ₆ , 500 MHz) of ruthmycin (1)	S17
Figure S14. Comparison of the experimental ECD and calculated ECD spectra	S18

Experimental Methods and Materials

General Experimental Procedures. UV spectra were recorded on a GE Ultraspec 8000 spectrophotometer (GE, Fairfield, CT), CD spectra were obtained on a Jasco J-810 spectropolarimeter (Jasco, Easton, MD), optical rotation was recorded on a Jasco DIP-370 Digital Polarimeter and IR spectra were measured on a Thermo Scientific Nicolet FT-IR 6700 spectrophotometer (Thermo Scientific, West Palm Beach, FL). All NMR data were recorded at 500 MHz and 400 MHz for ^1H , at 100 MHz for ^{13}C with Varian Inova NMR spectrometers (Agilent, Santa Clara, CA). LC-MS was conducted with a Waters 2695 LC module (Waters, Milford, MA). equipped with a micromass ZQ and a Symmetry Anal C_{18} column (4.6×250 mm, $5\ \mu\text{m}$; Waters, Milford, MA). HR-ESI-MS was accomplished using a Thermo Scientific Q Exactive mass spectrometer (Thermo Scientific, West Palm Beach, FL). HPLC analyses were performed on an Agilent 1260 system equipped with a photodiode array detector (PDA) detector and a Phenomenex C_{18} column (4.6×150 mm, $5\ \mu\text{m}$; Phenomenex, Torrance, CA). Semi-preparative HPLC separation was performed on a Varian Prostar 210 HPLC system equipped with a PDA detector 330 using a Supelco Discovery[®]Bio wide pore C_{18} column (250×21.2 mm, $10\ \mu\text{m}$; flow rate, 8 mL/min; Sigma-Aldrich, St. Louis, MO). All solvents used were of ACS grade and purchased from Pharmco-AAPER (Brookfield, CT). Sephadex LH-20 ($25 \sim 100\ \mu\text{m}$) was purchased from GE Healthcare (Little Chalfont, United Kingdom). C_{18} -functionalised silica gel ($40 \sim 63\ \mu\text{m}$) was purchased from Material Harvest Ltd. (Cambridge, United Kingdom). TLC silica gel plates (60 F_{254}) were purchased from EMD Chemicals Inc. (Darmstadt, Germany).

Fermentation, Extraction, Isolation and Purification. Isolation of *Streptomyces* sp. RM-4-15 and its phylogenetic characterization were described previously.^{S1} *Streptomyces* sp. RM-4-15 was cultivated on M_2 -agar (glucose, 10.0 g/L; malt extract, 10.0 g/L; yeast extract, 4.0 g/L; agar, 15.0 g/L) plates at $28\ ^\circ\text{C}$ for 7 days. Chunks of agar from the corresponding plate containing the fully-grown strain were used to inoculate ten 250 mL Erlenmeyer flasks, each containing 50 mL of medium A (soluble starch, 20.0 g/L; glucose, 10.0 g/L; peptone, 5.0 g/L; yeast extract, 5.0 g/L; NaCl , 4.0 g/L; K_2HPO_4 , 0.5 g/L; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g/L; CaCO_3 , 2.0 g/L). Cultures were grown at $28\ ^\circ\text{C}$ with shaking (210 rpm) for 3 days and used as seed for the corresponding production scale fermentation. An aliquot of seed culture (3 mL) was used to inoculate 300 Erlenmeyer flasks (250 mL), each containing 100 mL of medium A. The fermentation was continued for 7 days at $28\ ^\circ\text{C}$ with 200 rpm agitation. The culture was subsequently centrifuged at 5,000 rpm for 30 minutes and the corresponding supernatant was extracted with EtOAc (3×3000 mL). The recovered organics were evaporated *in vacuo* at $38\ ^\circ\text{C}$ to afford 4.3 g of crude extract. The corresponding pelleted mycelium was also extracted with EtOAc (3×500 mL) and then the recovered organics were evaporated *in vacuo* at $38\ ^\circ\text{C}$ to yield 3.2 g of crude mycelial extract.

HPLC and TLC analysis indicated an identical set of metabolites in both culture broth extract and mycelial extract. Therefore, all recovered crude extracts were combined (7.5 g) for further processing. The crude extract was first fractionated by C_{18} column chromatography (20×5 cm) eluted with 20% (1000 mL), 30% (1000 mL), 40% (2000 mL), 50% (1500 mL) and 100% CH_3CN -water (2000 mL) at a flow rate of 5 mL/min to yield five fractions, I-V. Fraction III (405 mg) was subjected to a Sephadex LH-20 column (60×2 cm), using methanol to elute compounds at the flow rate of 2 mL/min to get five fractions III01-III05 (50 mL for each fraction). Fraction III04 was further purified by using semi-preparative HPLC (Supelco Discovery[®]Bio wide pore C_{18} column, 250×21.2 mm, $10\ \mu\text{m}$; mobile phase: 35% CH_3CN /water; 8 mL/min; A_{340}) to yield compound **1** (5 mg, retention time: 16.3 min) as a yellow amorphous powder; $[\alpha]_{\text{D}}^{25} + 13.3^0$ (c 0.7, MeOH); UV (MeOH) λ_{max} ($\log \epsilon$) 342 nm (2.47); IR (KBr) ν_{max} 3374, 3177, 1717, 1680, 1607, 1334, 1121, 1068 cm^{-1} ; ^{13}C and ^1H NMR data, see Table 1;

APCI-MS: m/z 387.2 $[M + H]^+$, m/z 385.2 $[M - H]^-$ and m/z 771.6 $[2M - H]^-$; HR-ESI-MS: m/z 387.1437 (calcd for $C_{21}H_{23}O_7$, 387.1438).

Computational analysis. All the quantum chemical computations were performed with Gaussian 09 software (Gaussian, Inc., Pittsburgh, PA, 2009).^{S2} The structure of two enantiomers of **1** were optimized with the DFT method at the B3LYP/6-31G(d) level. The TDDFT (time-dependent density functional theory) was used to calculate the ECD (electronic circular dichroism) spectra of the two enantiomers of **1** at B3LYP/6-311G(d,p) level. The calculated ECD spectra were then generated by using GaussSum 2.2^{S3} at the wavelength range of 200nm - 400nm with a fwhm (full width at half maximum) for each peak set to 0.4 eV.

Antifungal Assays. *Saccharomyces cerevisiae* (ATCC 204508) was used in a broth microdilution antifungal assay. The assay was performed in a 96-well plate using the CLSI (formerly NCCLS) guidelines.^{S4} Compound **1** was dissolved in DMSO at a concentration of 10 mM and was serially diluted such that the final concentration ranges from 60 μ M-0.47 μ M. Individual colonies from an overnight plate were used to inoculate 5 mL YAPD (Bacto yeast extract, 10.0 g/L; adenine, 0.1 g/L; Bacto peptone, 20.0 g/L; glucose, 20.0 g/L) and incubated overnight at 37°C. The culture was diluted to OD₆₀₀ of 0.1 using sterile YAPD. A volume of 150 μ L of the diluted culture was transferred to each well. Amphotericin B and DMSO were used as positive and negative controls, respectively. Sample and controls were tested in triplicates. The plate was incubated at 37°C for 16 h with shaking (200 rpm) and the OD₆₀₀ was subsequently measured using a scanning microplate spectrofluorometer FLUOstar Omega (BMG Labtech, Cary, NC, USA). The acquired OD₆₀₀ values were normalized to the negative control wells (as 100% viability). The minimal concentration of **1** that caused growth inhibition was recorded as the MIC.

Antifungal activity against *Aspergillus nidulans* (ATCC 38163) was using a disc diffusion assay. Spores from each strain were suspended in 50 μ L YAPD broth and homogenously spread on V8 agar plates [V8 agar plate composition; 16.5 gm of calcium carbonate was mixed with 1 L of V8 juice. After centrifugation at $4,000 \times g$ for 10 min., the solution was adjusted to pH 7.0. The solution was diluted to 4 L with Milli-Q grade water, Bacto agar was added (1.3 % W/V) and the media was autoclaved]. A volume of 10 μ L of 10 mM DMSO stock of **1** (38.6 μ g), known antifungal agents amphotericin B (92.5 μ g) and cycloheximide (28.1 μ g), and 10 μ L of DMSO as negative control, were applied to sterile disks and allowed to air dry in a sterile environment. The dried disks were then transferred to the streaked plates and the plates were allowed to incubate in the dark for 3-4 days at 22°C and the inhibition zones were recorded (see Figure S1).

Antibacterial Assays. The protocol used for the determination of MIC was as that described previously with minor modifications.^{S5} Two bacterial strains, *Staphylococcus aureus* (ATCC 6538) and *Salmonella enterica* (ATCC 10708), were used as model strains for this assessment. Single colonies from each strain were grown in 5 mL of tryptic soy broth (BD 211825) and nutrient broth (BD 234000) media, respectively, and allowed to grow overnight. The overnight culture for each strain was diluted to OD₆₀₀ 0.8-1.1 and 150 μ L of the diluted culture were added to each well. The plate was incubated at 37°C for 16 h with shaking (150 rpm) and kanamycin and DMSO were used as positive and negative controls, respectively. Sample (final concentrations 1-120 μ M) and controls were tested in triplicates.

Cytotoxicity assays. A resazurin-based cytotoxicity assay, also known as AlamarBlue assay, was used to assess the cytotoxicity of agents against the human lung non-small cell carcinoma cell line A549 cell line and human prostate cancer cell lines PC3 where degree of cytotoxicity was based upon residual

metabolic activity as assessed via reduction of resazurin (7-hydroxy-10-oxido-phenoxazin-10-ium-3-one) to its fluorescent product resorufin. A549 and PC3 cells, purchased from ATCC (Manassas, VA, USA), were grown in DMEM/F-12 Kaighn's modification and MEM/EBSS media, respectively (Thermo scientific HyClone, Logan, UT, USA), with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine. Cells were seeded at a density of 2×10^3 cells per well onto 96-well culture plates with a clear bottom (Corning, NY, USA), incubated 24 hrs at 37 °C in a humidified atmosphere containing 5% CO₂ and were exposed to standard toxin (positive controls: 1.5 mM hydrogen peroxide, 10 µg/ml actinomycin D) and **1** for 2 days. Resazurin (150 µM final concentration) was subsequently added to each well and the plates were shaken briefly for 10 seconds and were incubated for another 3 h at 37 °C to allow viable cells to convert resazurin into resorufin. The fluorescence intensity for resorufin was detected on a scanning microplate spectrofluorometer FLUOstar Omega (BMG LABTECH GmbH, Ortenberg, Germany) using an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The assay was repeated in 3 independent experimental replications. In each replication, the emission of fluorescence of resorufin values in treated cells were normalized to, and expressed as a percent of, the mean resorufin emission values of positive control (untreated, metabolically active cells; 100%, all cells are viable).

Supplemental References

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- S5. Wiegand, I.; Hilpert, K.; Hancock, R. E. *Nat. Protoc.* **2008**, *3*, 163–175.

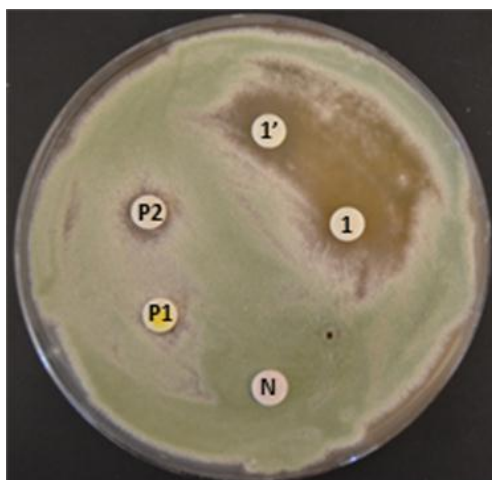
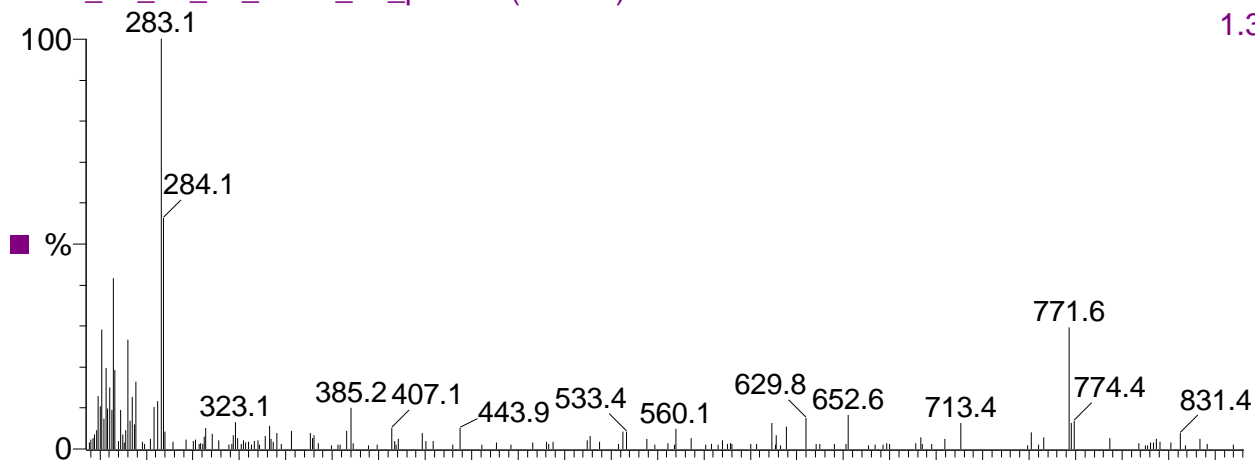


Figure S1. Antifungal disc diffusion assay using *Aspergillus nidulans* as the fungal indicator strain (P1, amphotericin B (92.5 μg); P2, cycloheximide (28.1 μg); N, DMSO; 1 (**1**, 38.6 μg) and 1' (**1**, 19.3 μg)).

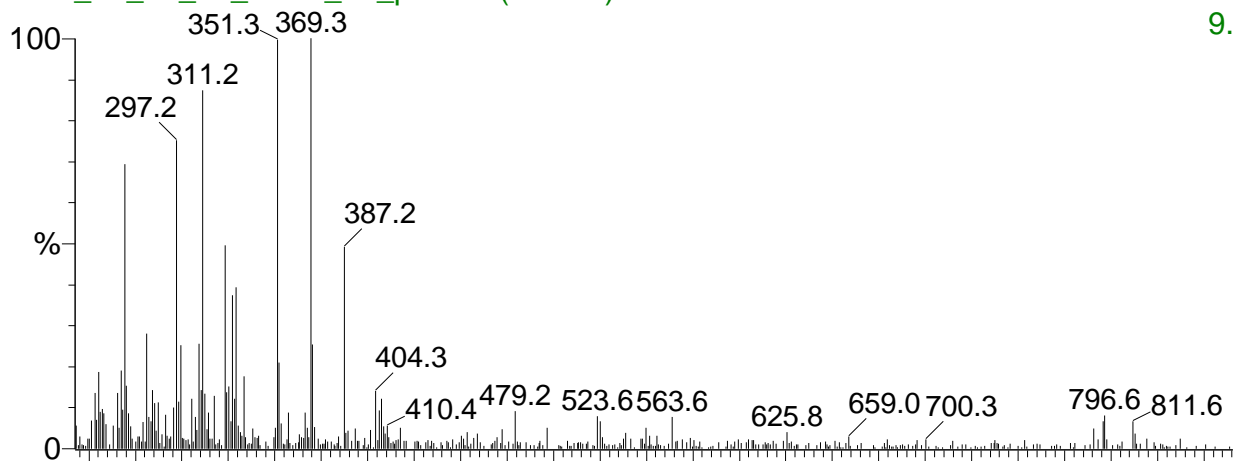
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2: Scan ES-
1.38e5



CPRI_07_27_12_FR21_24_p4 527 (21.235)

1: Scan ES+
9.27e5



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3: Diode Array
7.15e5

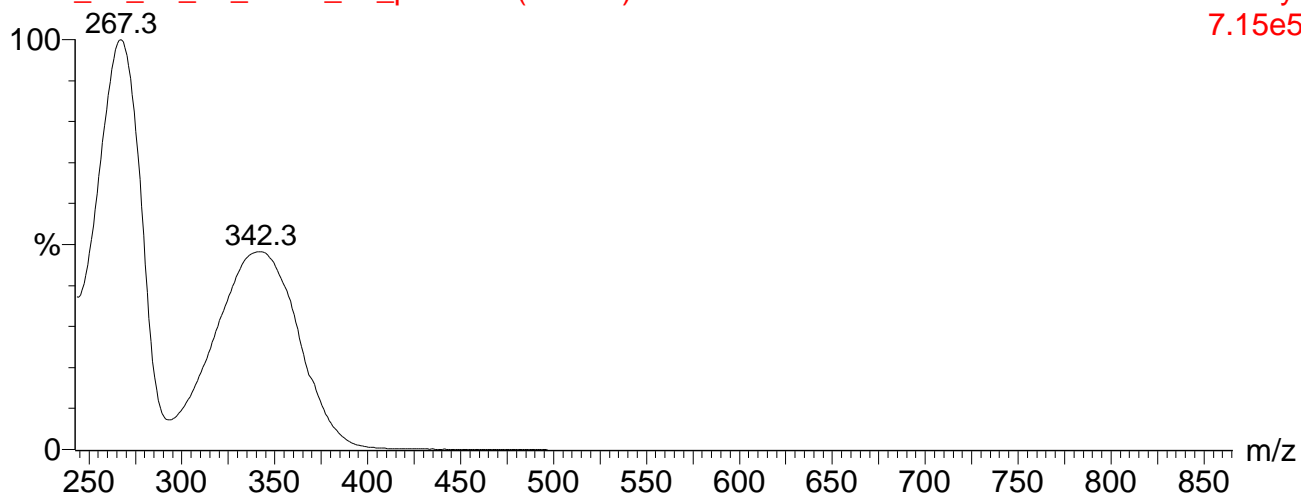


Figure S2. APCI-MS (positive and negative mode) of ruthmycin (**1**)

13-0170 #29-49 RT: 0.81-1.36 AV: 21 NL: 3.47E7
T: FTMS + p ESI Full lock ms [300.00-1000.00]

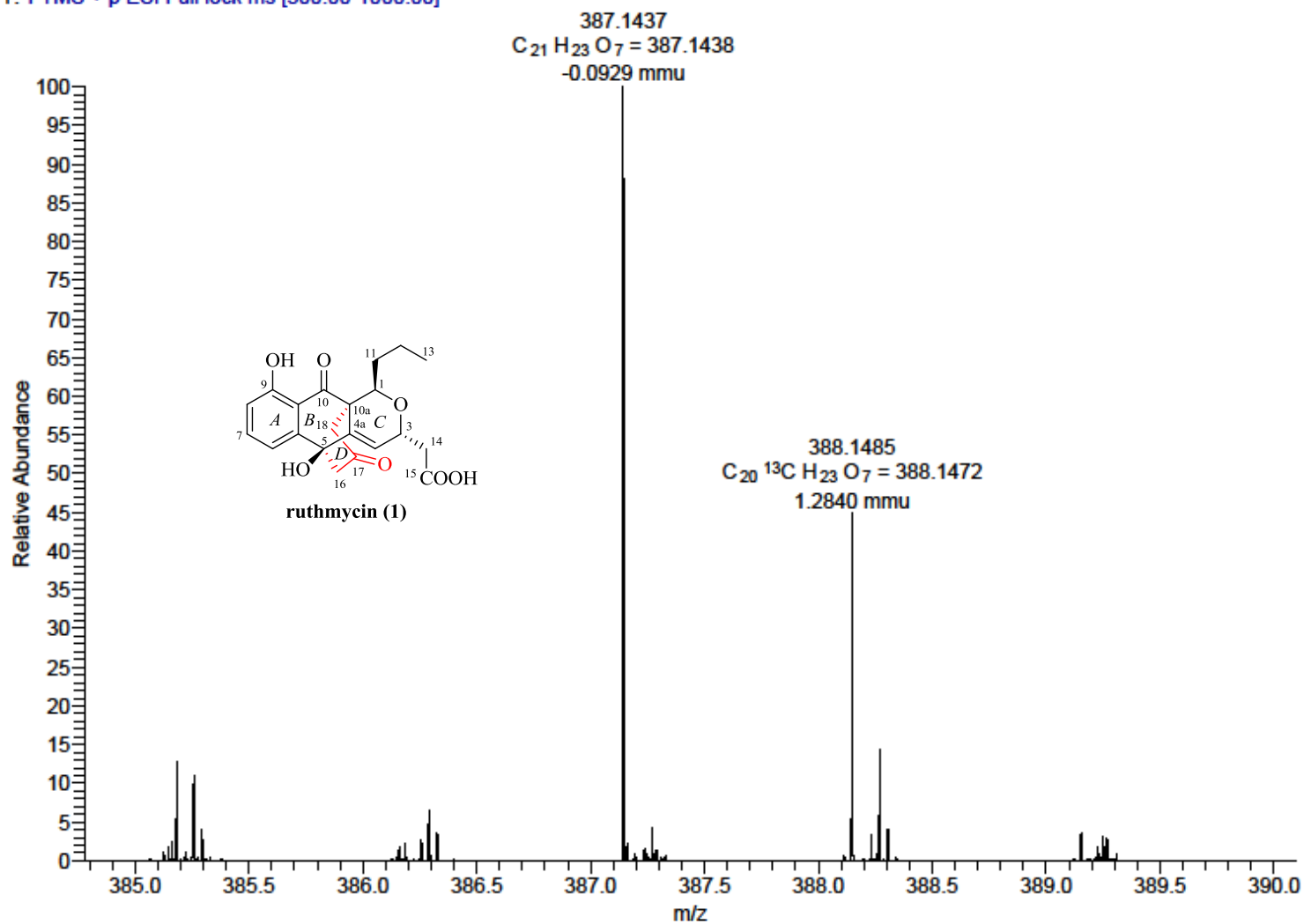


Figure S3. HR-ESI-MS (positive mode) of ruthmycin (1)

S8

RM-4-15-8 HNMR, DMSO-d₆, 500MHz

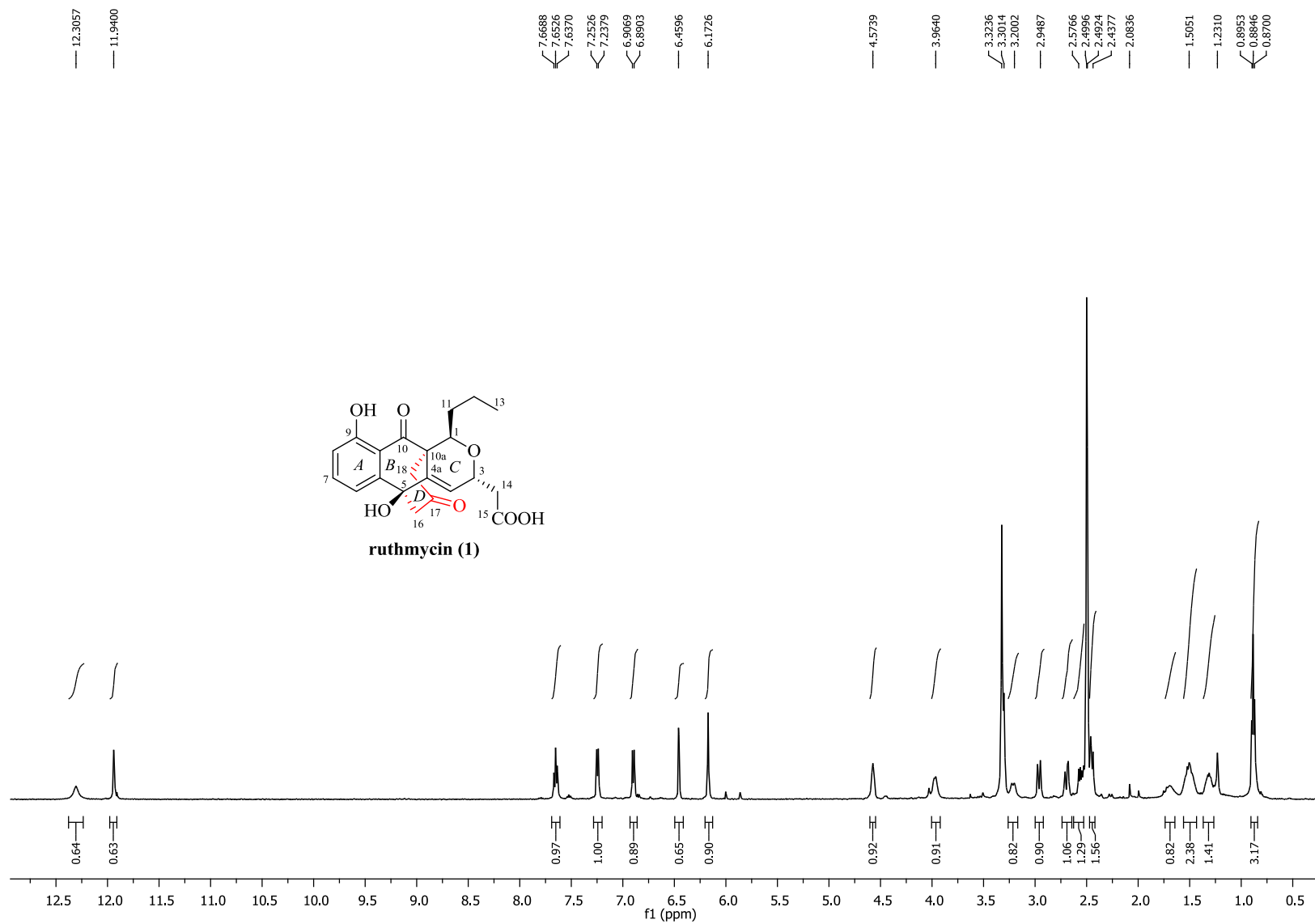


Figure S5. ¹H-NMR (DMSO-d₆, 500 MHz) of ruthmycin (**1**)

RM-4-15-8 HNMR, CDCl₃, 500MHz

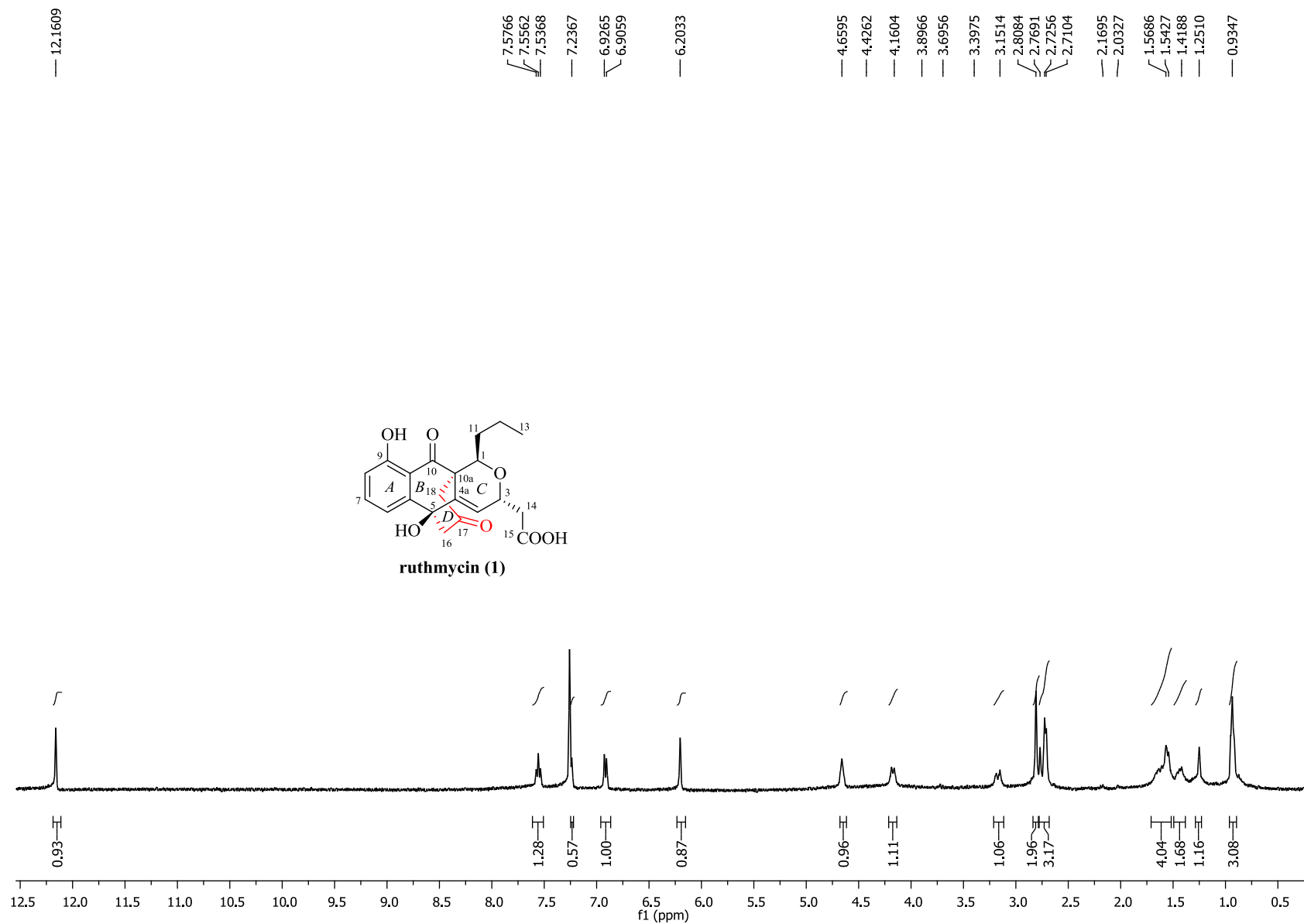


Figure S6. ¹H-NMR (CDCl₃, 500 MHz) of ruthmycin (1)

RM-4-15-8 HNMR, CD₃OD, 500MHz

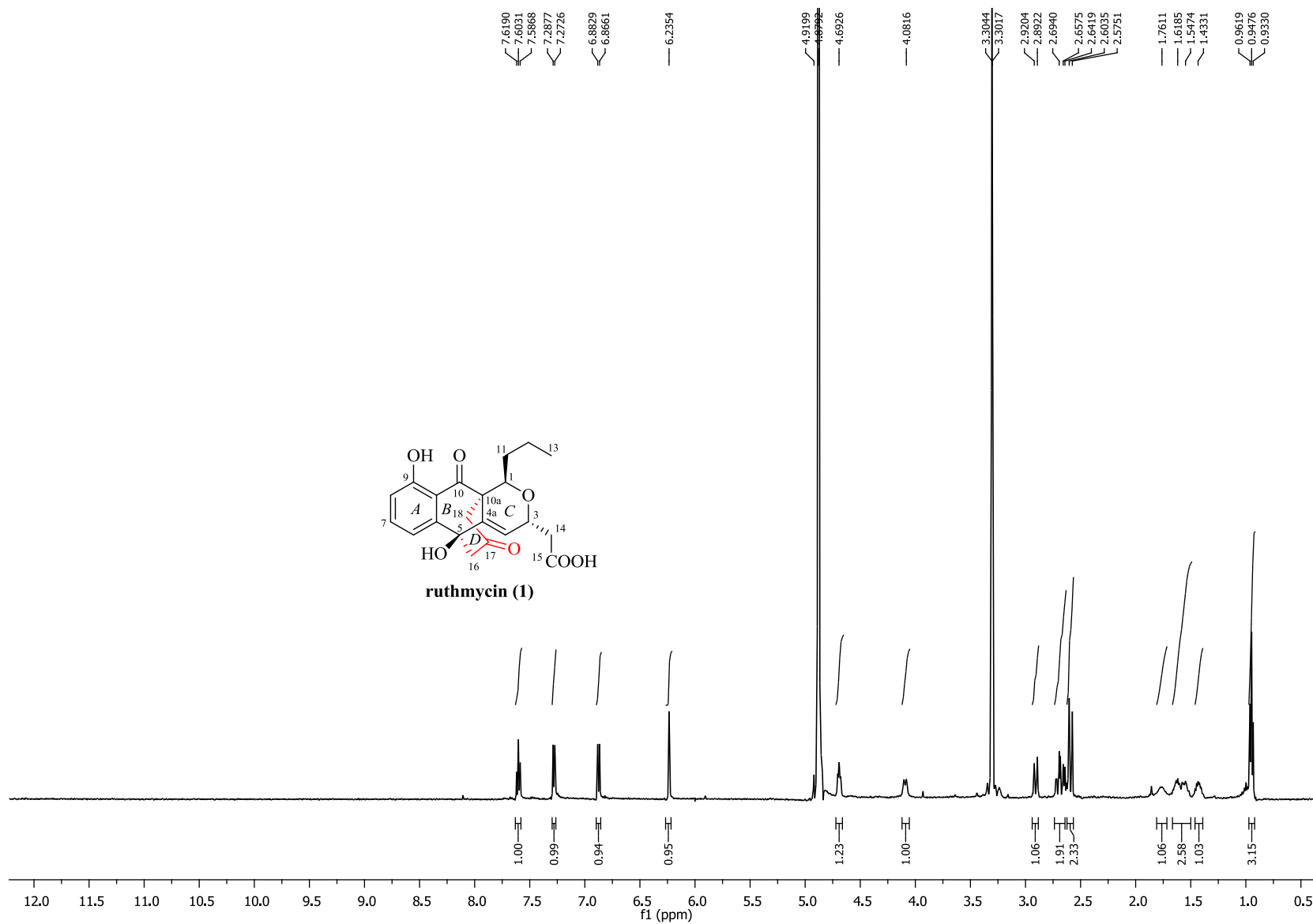


Figure S7. ¹H-NMR (CD₃OD, 500 MHz) of ruthmycin (1)

RM-4-15-8 CNMR, 100 MHz, Acetone-d₆

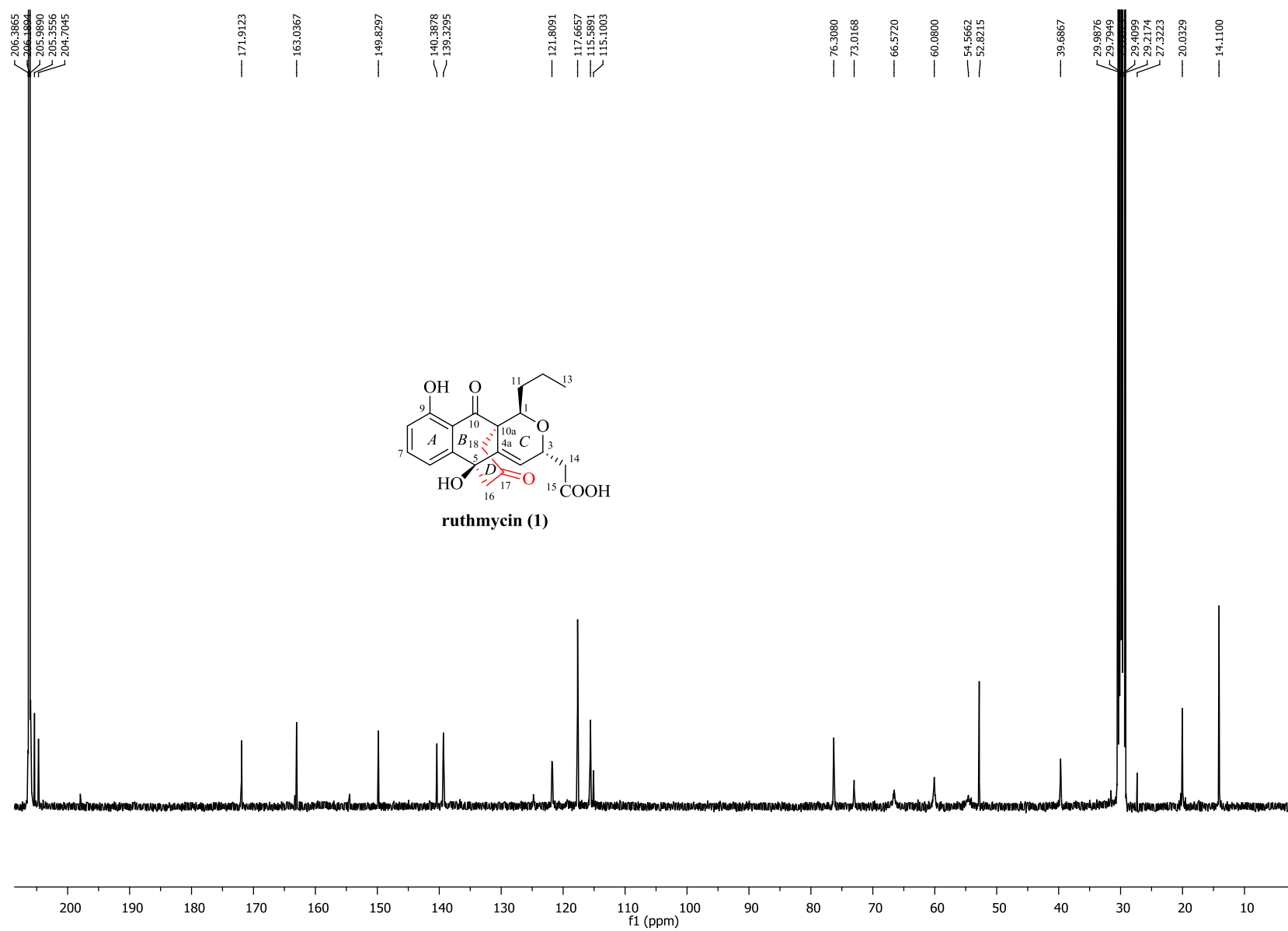
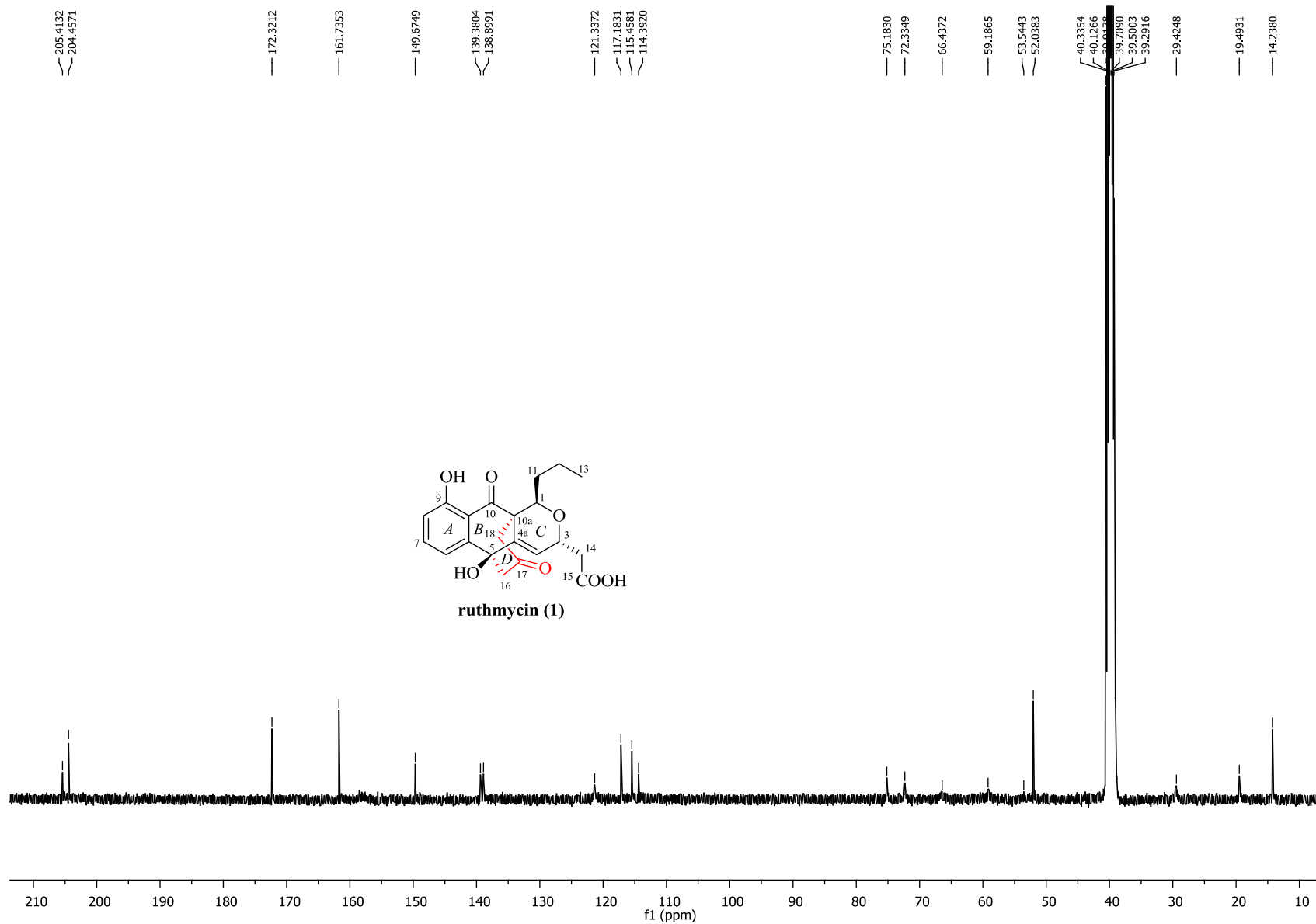


Figure S8. ¹³C-NMR (Acetone-d₆, 100 MHz) of ruthmycin (1)

RM-4-15-8 CNMR, DMSO-*d*₆, 100MHz



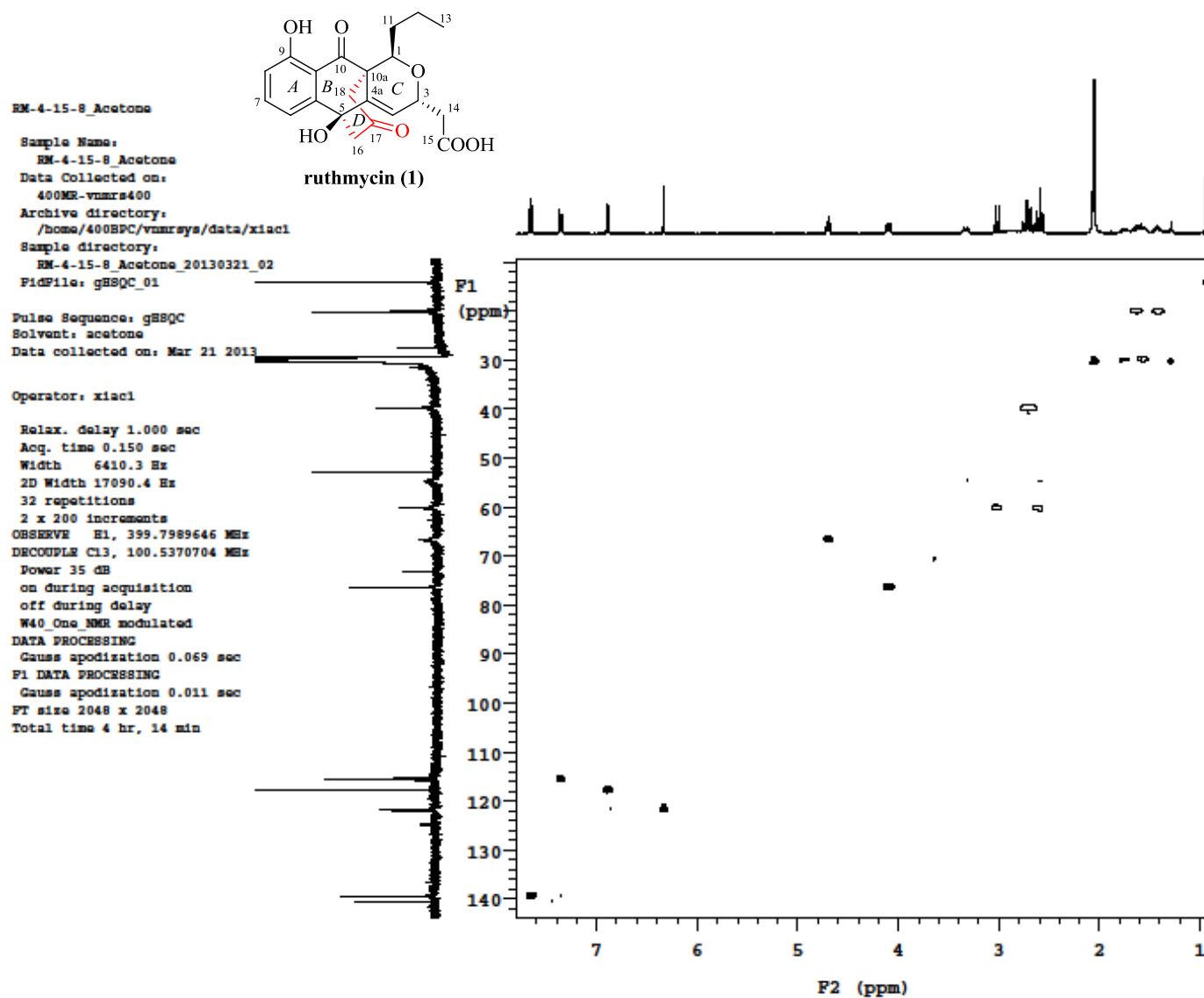


Figure S10. HSQC spectrum (Acetone- d_6 , 400 MHz) of ruthmycin (1)

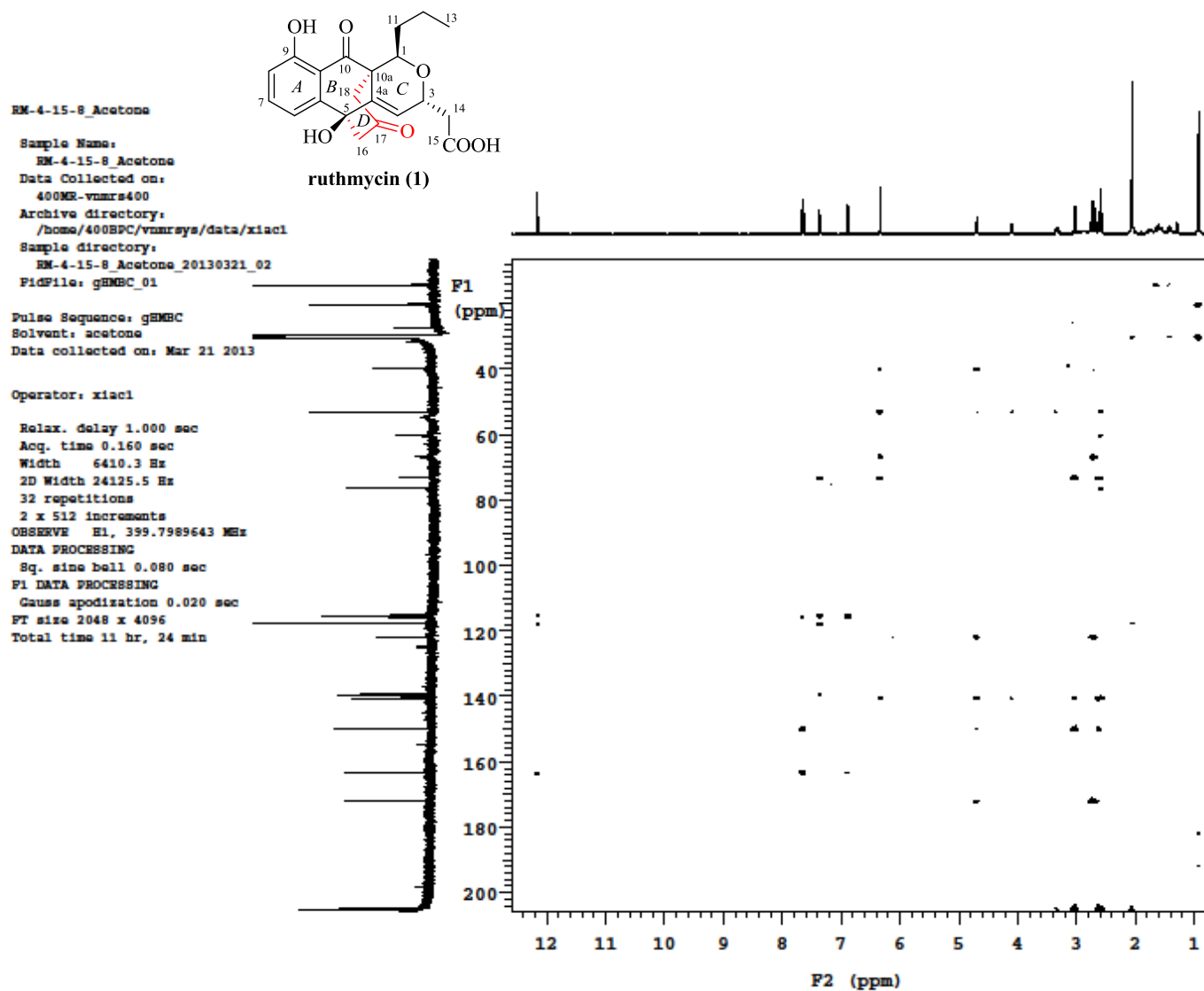


Figure S11. HMBC spectrum (Acetone- d_6 , 400 MHz) of ruthmycin (1)

FM 1 2 8 F0F Acetone-d6

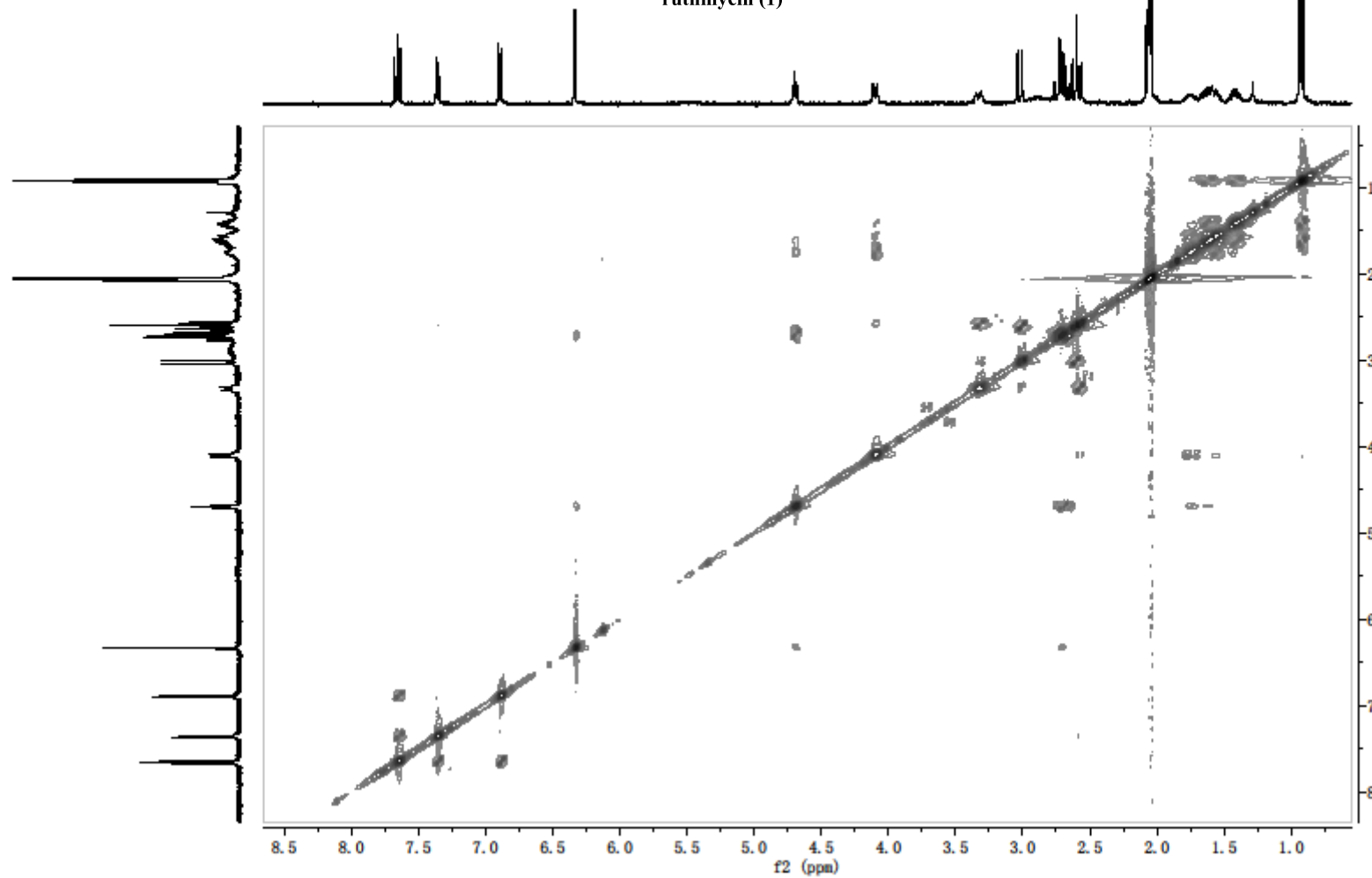
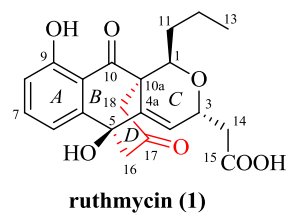


Figure S12. ROESY spectrum (Acetone-*d*₆, 400 MHz) of ruthmycin (**1**)

RM-4-15-8 ROESY

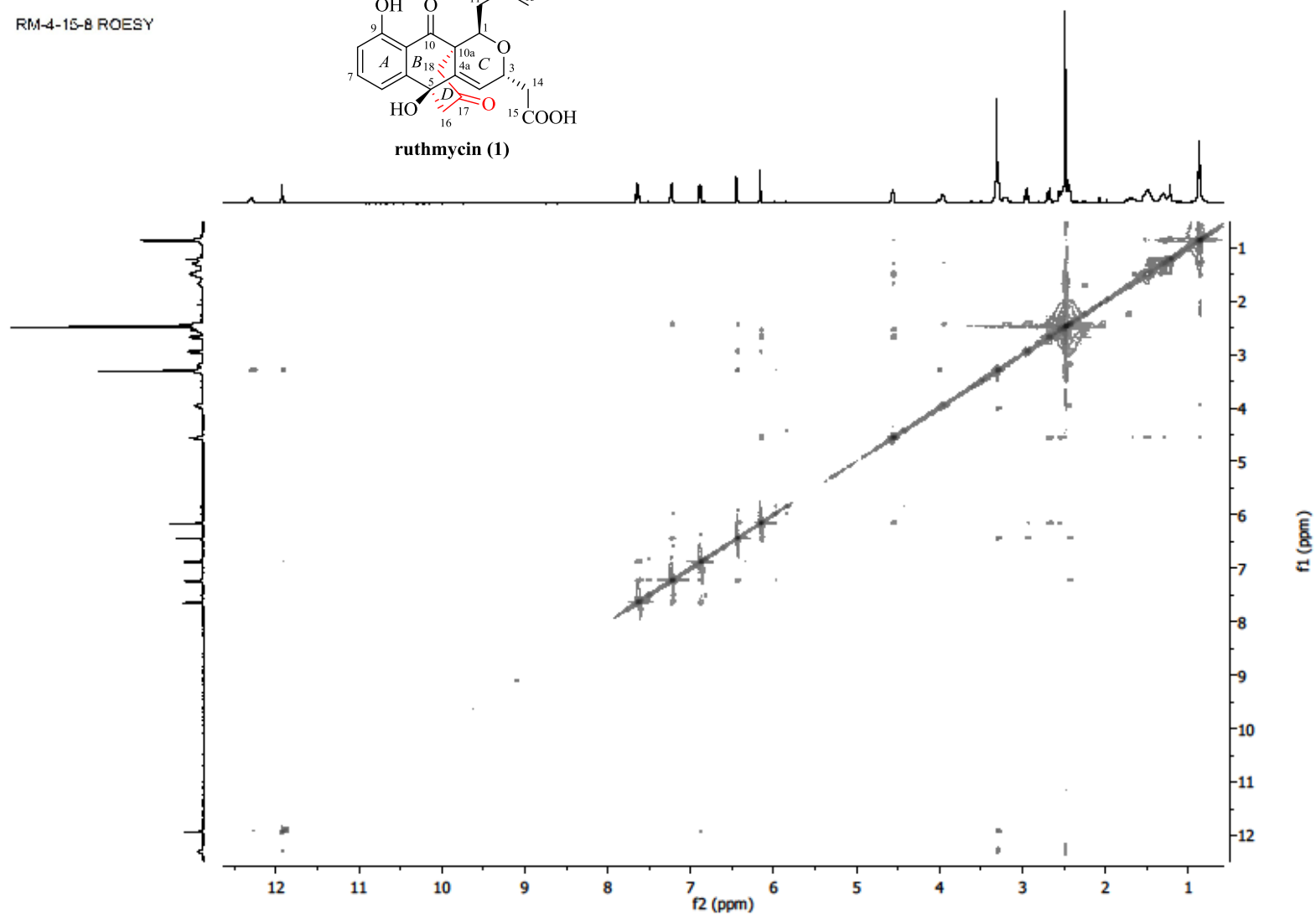
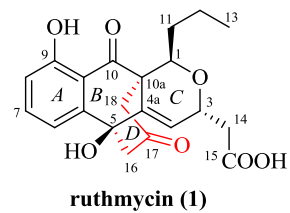


Figure S13. ROESY spectrum (DMSO- d_6 , 500 MHz) of ruthmycin (**1**)

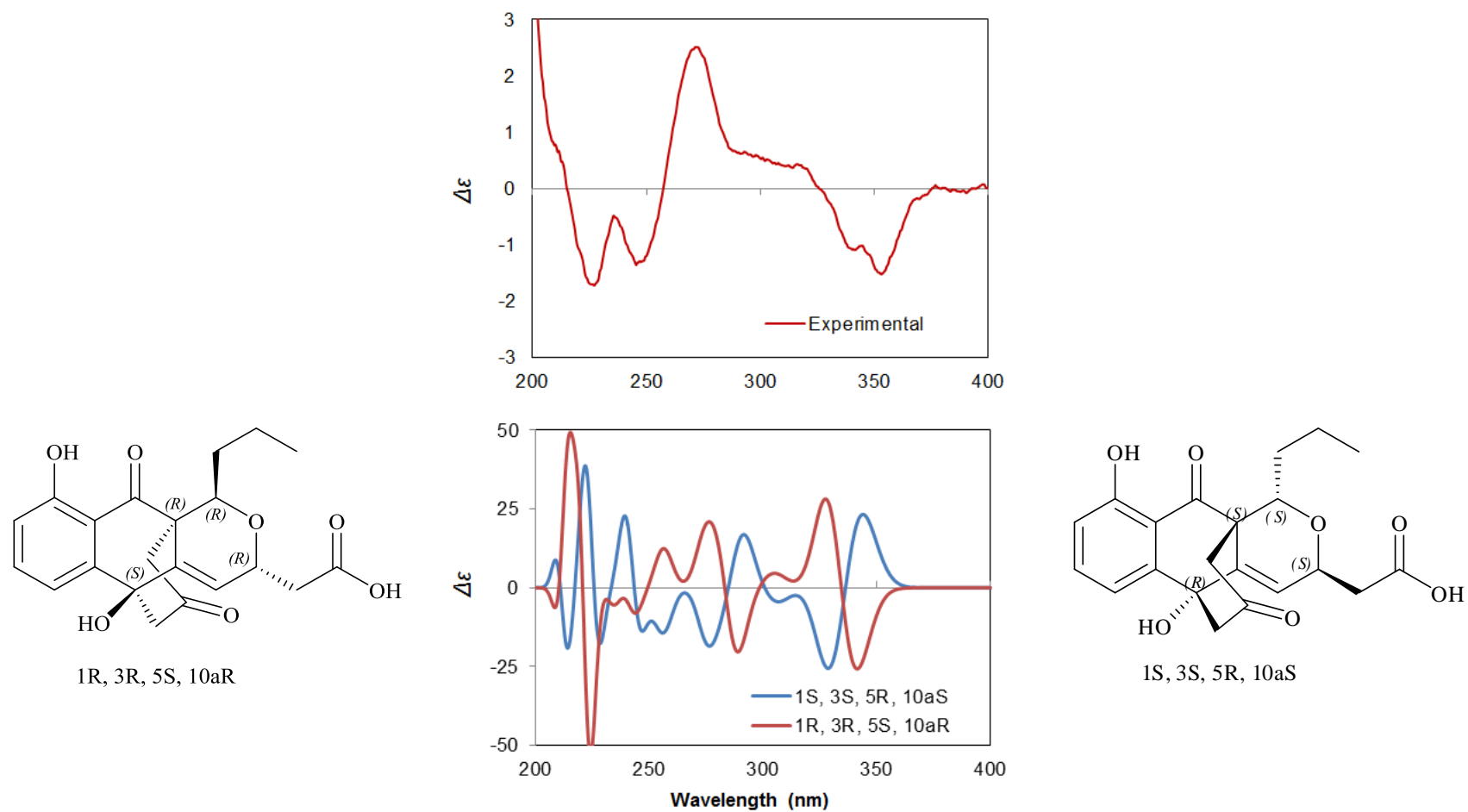


Figure S14. Comparison of the experimental ECD and two calculated ECD spectra of two probable configurations of ruthmycin (**1**)